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UNITED STATES PATENT APPLICATION

OF

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FOR

CHIMERIC GFP-AEQUORIN AS BIOLUMINESCENT Ca^{++} REPORTERS AT THE SINGLE CELL LEVEL

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application is based on and claims the benefit of U.S. Provisional Application Nos. 60/208,314, filed June 1, 2000 (Attorney Docket No. 03495.6051), 60/210,526, filed June 6, 2000 (Attorney Docket No. 03495.6052), and 60/255,111, filed December 14, 2000 (Attorney Docket No. 03495.6059). The entire disclosure of each of these applications is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

[002] This invention relates to a modified bioluminescent system comprising a flourescent molecule covalently linked with a photoprotein allowing the transfer of energy by Chemiluminescence Resonance Energy Transfer (CRET). This invention also relates to the use of the modified bioluminescent system in *in vivo* and *in vitro* assays.

[003] Calcium is implicated in the regulation of a great variety of intracellular processes (1). Several techniques are most commonly used for intracellular Ca^{++} monitoring. Patch-clamp and Ca^{++} selective microelectrodes give cumulative measurements of Ca^{++} fluxes in a restricted number of cells. On the other hand, intracellular [Ca^{++}] dynamics in large populations of cells can be visualized with fluorescent probes (2). Genetic tools could provide new methods for Ca^{++} monitoring.

[004] Two groups of genetic Ca^{++} probes are at present available. The first category uses the principle of Fluorescence Resonance Energy Transfer (FRET) between two variants of the green fluorescent protein (GFP). The two GFP are covalently linked by a calmodulin binding sequence alone or in combination with calmodulin so that intramolecular FRET does (3) or does not (4) occur in response to Ca^{++} influx. The second category is composed by bioluminescent proteins, such as aequorin (5, 6). The active protein is formed in the presence of molecular oxygen from apoaequorin (189 amino acids) and its luciferin, coelenterazine (Mr 423) (7).

[005] The binding of Ca^{++} to aequorin, which has three EF-hand structures characteristic of Ca^{++} binding sites, induces a conformational change resulting in the oxidation of coelenterazine *via* an intramolecular reaction. Moreover, the coelenteramide so produced is in an excited state, and blue light (max: 470nm) is emitted when it returns to its ground state (8). Such a bioluminescent genetic marker presents the advantage over Ca^{++}-sensitive fluorescent dyes of being easily targeted to specific cells and in subcellular compartments with appropriate regulatory elements and

peptide signals (9). The bioluminescent process does not require light excitation like fluorescent probes or proteins, and thus does not induce autofluorescence, photobleaching and biological degradation problems. Furthermore, aequorin is not toxic, does not bind other divalent cations and does not interfere with the $[Ca^{++}]_i$ buffer system even when microinjected at high concentrations. Its low affinity for Ca^{++} (Kd = 10 (μM) is probably responsible for this and makes aequorin a good sensor in the range of biological $[Ca^{++}]$ variations.

[006] Although providing a good ratio of signal over background, aequorin signals are very difficult to detect because of aequorin's low light quantum yield, that is, the number of emitted photons per protein that bind Ca^{++}. In the jellyfish, *Aequorea victoria*, from which aequorin has been isolated (10), the protein is associated with the GFP (11). After Ca^{++} binding, the energy acquired by aequorin is transferred from the activated oxyluciferin to GFP without emission of blue light. The GFP acceptor fluorophore is excited by the oxycoelenterazine through a radiationless energy transfer. Then, a green light (max, 509nm) is emitted when the excited GFP returns to its ground state (12).

[007] Such intermolecular radiationless energy transfer is not unusual in bioluminescence and has already been shown to increase the quantum yield of the bioluminescent process in *Renilla*, another coelenterate (13). The gain measured *in vitro* ranges from 3 to 5 fold (14). It is possible to reconstitute *in vitro* the *Renilla* system and obtain the spectral shift with low equimolar concentrations of its components because the luciferase and the green fluorescent protein bind together (14).

[008] In the *Aequorea* system, binding between purified photoprotein and GFP does not occur in solution, even when present at high concentrations (15). *In vivo*, energy transfer occurs because of the high concentration of GFP. It can be obtained *in vitro* through the co-adsorption of aequorin and GFP on DEAE cellulose membranes (15). The Förster equation shows that the efficiency of this process depends on several conditions described in the case of FRET. The emission spectrum of the donor must have the greatest overlap with the excitation spectrum of the acceptor. The energy transferred is also strongly dependent on the geometry, in particular, the relative orientation and distance of the two dipoles and modulated by their respective motion (16).

[009] An aim of this invention is to develop a dual reporter gene combining properties of Ca^{++} -sensitivity and fluorescence of aequorin and GFP, respectively. The fusion protein, which can be detected with classical epifluorescence, can be used to monitor calcium activities. The configuration of the molecules of the invention increases their overall turnover and allows an efficient intramolecular Chemiluminescence Resonance Energy Transfer (CRET). As a result, the quantum yield of aequorin appears to be higher. This invention shows that physiological calcium signals can be visualized in single eukaryotic cells with an intensified CCD camera. Other constructs described here target the fusion protein to the neurite membrane.

SUMMARY OF THE INVENTION

[010] This invention thus provides a modified bioluminescent system comprising a fluorescent molecule covalently linked with a photoprotein, wherein the link between the two proteins has the function to stabilize the modified bioluminescent system and allow the transfer of the energy by Chemiluminescence Resonance Energy Transfer (CRET). In a preferred embodiment, the bioluminescent system comprises an GFP protein covalently linked to a aequorin protein, wherein the link between the two proteins has the function to stabilize the modified bioluminescent system and to allow the transfer of the energy by Chemiluminescence Resonance Energy Transfer (CRET).

[011] In one embodiment of a modified bioluminescent system according to the invention, the bioluminescent system comprises an GFP protein covalently linked to an aequorin protein, wherein the link between the two proteins is constituted by at least 5 amino acids and optionally at least 5 amino acids and at least one copy of 9 amino acids. The link has the function of stabilizing the system and allowing the transfer of energy by Chemiluminescence Resonance Energy Transfer (CRET).

[012] In a preferred embodiment, the bioluminescent system comprises a GFP protein covalently linked to an aequorin protein, wherein the link between the two proteins is preferably constituted by at least 5 amino acids and five copies of 9 amino acids and has the function of stabilizing the system and allowing the transfer of energy by Chemiluminescence Resonance Energy Transfer (CRET).

[013] The two proteins can be separate or together functional. In addition, the modified bioluminescent system can be calcium sensitive and/or light sensitive.

[014] This invention also provides a method of screening *in vitro* a change in a physical, chemical, biochemical, or biological condition. The method comprises:

a) providing in different samples a bioluminescent system according to the invention in a reaction system containing an analyte of interest;

b) measuring whether light is produced; and

c) detecting a change based on the production of light.

[015] Further, this invention provides a method of screening *in vivo* a change in a physical, chemical, biochemical, or biological condition. The method comprises the steps of:

a) administering to a mammal an acceptable composition comprising a bioluminescent system according to the invention;

b) detecting whether light is produced; and

c) optionally measuring ionic concentration of calcium flux.

[016] In addition, this invention provides a composition comprising a purified polypeptide, wherein the composition has the functional characteristics of binding calcium ions and permitting measureable energy, said energy depending of the quantity of calcium bound and of the quantity of polypeptides in said composition in absence of any light excitation.

[017] In addition, this invention provides a purified polypeptide having the amino acid sequence of SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; SEQ ID NO: 5; and SEQ ID NO: 6.

[018] In other embodiments, this invention provides a polynucleotide having the sequence of SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; and SEQ ID NO: 12.

[019] This invention also provides a culture as deposited at the C.N.C.M. and containing the plasmid No. I-2507; the plasmid No. I-2508; the plasmid No. I-2509; the plasmid No. I-2510; the plasmid No. I-2511; the plasmid No. I-2512; or the plasmid No. I-2513.

[020] Further, this invention provides a peptide linker having the function after translation to approach a donor site to an acceptor site in optimal conditions to permit a direct transfer of energy by chemiluminescence in a purified polypeptide according to the invention. The nucleotide linker can have, for example, the nucleotide sequence of SEQ ID No: 13; SEQ ID No: 14; SEQ ID No: 15; SEQ ID No: 16, or SEQ ID No: 17. The peptide linker can comprise at least 5 amino acids and comprising the amino acid sequence of SEQ ID No: 18; SEQ ID No: 19; SEQ ID No: 20; SEQ ID No: 21, or SEQ ID No: 22.

[021] A kit for measuring the transfer of energy *in vivo* or *in vitro* contains at least one of the polypeptides according to the invention or the polynucleotide according to the invention and the reagents necessary for visualizing or detecting the said transfer in presence or in absence of a molecule of interest.

[022] In another embodiment, the invention provides a fusion protein of the formula:

GFP - LINKER - AEQ;

[023] wherein GFP is green fluorescent protein; AEQ is aequorin; and LINKER is a polypeptide of 4-63 amino acids, preferably 14-50 amino acids.

[024] The LINKER can comprise the following amino acids:

[025] (Gly Gly Ser Gly Ser Gly Gly Gln Ser [SEQ ID NO: 25])$_n$, wherein n is 1-5. Preferably n is 1 or n is 5. LINKER can also include the amino acid sequence Ser Gly Leu Arg Ser [SEQ ID NO: 26].

[026] Another fusion protein for energy transfer from aequorin to green fluorescent protein by Chemiluminescence Resonance Energy Transfer (CRET) following activation of the aequorin in the presence of Ca^{++} has the formula:

GFP - LINKER - AEQ;

[027] wherein GFP is green fluorescent protein; AEQ is aequorin; and LINKER comprises the following amino acids:

[028] (Gly Gly Ser Gly Ser Gly Gly Gln Ser [SEQ ID NO: 25])$_n$, wherein n is 1-5; and wherein the fusion protein has an affinity for Ca^{++} ions and a half-life of at least 24 hours. The LINKER can include the amino acid sequence Ser Gly Leu Arg Ser [SEQ ID NO: 26]. In addition, the fusion protein can further comprise a peptide signal sequence for targeting the fusion protein to a cell or to a subcellular compartment.

[029] This invention also provides polynucleotides encoding fusion proteins as described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[030] This invention will be described with reference to the drawings in which:

[031] Fig. 1 is a schematic map of different constructions. All the constructs were under the control of the human cytomegalovirus promoter (PCMV). An asterisk indicates the position of a Val-163-Ala mutation. In pGA, the coding sequences of GFP and aequorin are separated by 5 codons. One to five linkers (in brackets) have been added in pG$_i$A where i is the number of linker. Linkers were oriented so as to encode a

9 amino acid repeat. Complete Synaptotagmin 1 or its transmembrane part (tSyn) were fused in frame with the G5A.

[032] Fig. 2 depicts Ca^{++} CRET activities on cellular extracts. Emission spectra of aequorin and several GFP-Aequorin fusion proteins were calibrated as a percentage of maximum intensity. CRET measurements are expressed as the ratio of green (500nm) over blue (450nm) photons.

[033] Fig. 3 depicts GFP fluorescence of GFP-Apoaequorin proteins in Neuro2A cells transfected with pGm (A), pGA (B), pG2A (C), and pG5A (D). Confocal superposition of GFP fluorescence and immunostaining of synaptotagmin in cells expressing either pSG5A (E) or pStG5A (F) is shown.

[034] Fig. 4 depicts Ca^{++}-induced bioluminescence detected at the single cell level. Neuro2A cells transfected with pGA (A.1-4) or pSG5A (B) were pre-incubated with 5μM coelenterazine in a Ca^{++}-free buffer. (A.3) GFP Fluorescence made it possible to choose transfected cells. The background recorded before $CaC1_2$ addition (A.2) corresponds to the relative light unit (RLU) level at time 0 of experiment (A.4, B). Intensities of fluorescence and bioluminescence activity are translated in scaled pseudocolors. Representative pictures of the chosen field are shown after addition of 5mM $CaCl_2$ and 5μM A23187 at 13 sec. and 159 sec, respectively, after the beginning of the acquisition (A.1). (A.4) Each profile indicates the intensity of light emitted by a single cell.

[035] Five regions of interest were defined by encircling individual cell soma. With pGA (data not shown) or pSG5A (B) transfection, a high concentration of $CaCl_2$, (100mM) was added at the end of the experiment (500sec.) to check that the bioluminescent protein was still active. (C) Control experiments were made with Fluo-3 AM on mock-transfected Neuro2A cells.

[036] Fig. 5 depicts the results of analysis of protein stability for various fusion proteins.

[037] Fig. 6 depicts the results of the determination of the Ca^{++} affinity of aequorin and fusion protein G5A.

[038] Fig. 7 Calibration curves between the bioluminescent activity and Ca2+.

[039] Fig. 8 Fluorescence and Ca2+-induced bioluminescent activity in dissociated neurons in culture infected with adenoviral-G5A vectors.

[040] Fig. 9 Fluorescence and Ca2+-induced bioluminescent activities in dissociated neurons in culture infected with adenoviral-SG5A vectors.

[041] Fig. 10 shows representative pattern of luminescence activity after injection of GA plasmid at the one cell stage of *Xenopus* embryo.

[042] Fig. 11 shows a transgenic *Xenopus* larva with GFP-aequorin.

DETAILED DESCRIPTION OF THE INVENTION

[043] Among the coelenterates, bioluminescent species exist. Numerous studies have shown that the bioluminescence is generated by photoproteins that are sensitive to calcium. These proteins emit a flash of light in response to an increase in the concentration of calcium ions. Among these photoproteins, aequorin is one of the most well studied (Blinks *et al.*, 1976).

[044] Isolated in the jellyfish, *Aequoria victoria* (Shimomura *et al.*, 1962), aequorin, after binding with three calcium ions, emits a flash of blue light with a spectrum of maximum wavelength 470 nm. Contrary to a classical luciferase-luciferin reaction, the emission of light does not require oxygen, and the total amount of light is proportional to the amount of protein. Oxygen is necessary, however, to reconstitute the aequorin, by the action of apoaequorin, a protein with a molecular mass of 21kDa, and coelenterazine. The emission of photons is caused by a peroxidation reaction in the coelenterazine, after binding with the three calcium ions on the aequorin. Two hypotheses have been suggested for this process: (i) the binding between aequorin and calcium ions induces the emission of light by a conformational change in the protein, allowing oxygen to react with coelenterazine, and (ii) oxygen plays a role in the binding between coelenterazine and apoaequorin (Shimomura and Johnson, 1978). Aequorin may be recreated *in vitro* and *in vivo* by eliminating oxyluciferin, adding luciferin (coelenterazine) in the presence of β-mercaptoethanol and oxygen (Shimomura and Johnson, 1978). The necessity of adding β-mercaptoethanol or a reducing agent to reconstitute aequorin is presumably due to the presence of at least one sulfhydryl group of cysteine 145 included in a negatively charged microenvironment (Charbonneau *et al.*, 1985).

[045] More than thirty semi-synthetic aequorins having different affinities for calcium ions have been characterized, based on the type of coelenterazine that binds to the protein (Shimomura, 1991; incorporated by reference herein). The dissociation constant between aequorin and the calcium ions is estimated to be between 0.1 mM (Allen *et al.*, 1997) and 1 mM (Prasher *et al.*, 1985). Although the relationship between light emission and calcium ion concentration may not be linear, a logarithmic relationship between the emission of light and the calcium ion concentration has

nonetheless been determined (Johnson and Shimomura, 1978). Indeed, a 200-fold increase in the signal to background noise ratio is measured when the Ca^{++} concentration goes from 10^{-7}M to 10^{-6}M , and by a factor of 1000, from 10^{-6}M to 10^{-5}M (Cobbold and Rink, 1987). Moreover, the kinetics of the signal emission is rapid enough to detect transitory increases in Ca^{++} ion concentrations. An increase in light intensity with a time constant of 6 msec, under calcium saturation conditions, has been shown (Blinks *et al.*, 1978). Aequorin is thus a photoprotein that is well adapted to measure rapid and elevated increases in Ca^{++} ions under physiological conditions.

[046] The cloning of the apoaequorin gene by Prasher *et al.*, (1985) and Inouye *et al.* (1985) has led to the creation of expression vectors, making possible its targeting in a specific cell compartment by fusion with nuclear, cytoplasmic, mitochondrial, endoplasmic reticulum, or plasma membrane signal peptides (Kendall *et al.*, 1992; Di Giorgio *et al.*, 1996). In addition, the *in vivo* expression of the protein makes possible its detection at low levels, leaving the intracellular physiology of calcium undisturbed.

[047] In nature, photoprotein activity is very often linked to a second protein. The most common is the "green flourescent protein" or GFP. The light emitted in this case is in fact green. The hypothesis of an energy transfer between aequorin and GFP by a radiative mechanism was proposed in the 1960s by Johnson *et al.*, (1962). The blue light emitted by aequorin in the presence of Ca^{++} is presumably absorbed by GFP and reemitted with a spectrum having a maximum wave length of 509 nm. other studies have shown that this transfer of energy occurs through a non-radiative mechanism made possible through the formation of heterotetramer between GFP and aequorin. Morise *et al.* (1974) have succeeded in visualizing this energy transfer *in vitro*, and a co-adsorption of the two molecules on a DEAE-cellulose membrane facilitates the process. Through this mechanism, it thus appears possible to increase the quantum efficiency of the system (Ward and Cormier, 1976).

[048] GFP, also isolated in the jelly fish *Aequoria victoria*, was recently cloned (Prasher *et al.*, 1992). It has been used in different biological systems as a cellular expression and lineage marker (Cubitt *et al.*, 1995). Detecting this protein using classical fluorescence microscopy is relatively easy to do in both living organisms and fixed tissue. In addition, fluorescent emission does not require the addition of a cofactor or coenzyme and depends on an autocatalytic post-translational process. The fluorophore, consisting of nine amino acids, is characterized by the formation of a cycle between serine 65 and glycine 67, which gives rise to an intermediate imidazolidine 5,

followed by oxidation of tyrosine 66, transforming it into dehydrotyrosine (Heim *et al.*, 1994). This group is found inside a cylinder composed of 11 β layers, which constitutes an environment that interacts directly with the chromophore (Yang *et al.*, 1996).

[049] Monitoring calcium fluxes in real time could help to understand the development, the plasticity, and the functioning of the central nervous system. In jellyfish, the chemiluminescent, calcium binding, aequorin protein is associated with the green fluorescent protein (GFP), and a green bioluminescent signal is emitted upon Ca^{++} stimulation. Aequorin alone is difficult to detect on the cellular and subcellular level owing to the weak emission of photons after excitation.

[050] The development of a new marker sensitive to calcium with a higher quantum yield was therefore initiated. This invention utilizes Chemiluminescence Resonance Energy Transfer (CRET) between the two molecules. Calcium sensitive bioluminescent reporter genes have been constructed by fusing GFP and aequorin resulting in much more light being emitted.

[051] Chemiluminescent and fluorescent activities of these fusion proteins have been assessed in mammalian cells. Cystosolic Ca^{++} increases were imaged at the single cell level with a cooled intensified CCD (coupled charge device) camera. This bifunctional reporter gene should allow the investigation of calcium activities in neuronal networks and in specific subcellular compartments in transgenic animals.

[052] GFP-aequorin Fusion Proteins as Ca^{++}-Activated Reporter Genes.

[053] According to this invention, a fusion protein has been constructed with aequorin and GFP to increase the quantum yield of Ca^{++}-induced bioluminescence. This activity can not be increased simply by co-expressing GFP with aequorin (data not shown). A thermoresistant GFP (Gm) was fused in frame with the NH_2 terminal region of apoaequorin (Fig. 1), since the C-terminal proline residue has been shown to be implicated in the Ca^{++}–activated bioluminescent process (20).

[054] Different constructs have been made with increasing size of linker between GFP and apoaequorin. The shortest spacer is formed by 5 amino acids and the longest by 50 amino acids (Fig. 1). All the fusion proteins showed a better Ca^{++}-triggered bioluminescent activity than aequorin alone. The increases of light emitting activity ranged from 19 to 65 times (Table 1) possibly because of greater protein stability.

TABLE 1
CA++ INDUCED CHEMILUMINESCENCE ACTIVITIES

Name	Mean ± SEM* RLU x 10^6 / 10 Uβgal
pA	0.15 (0.10; 021)
pGa	10.01 ± 4.4
pGlA	2.96 (3.39; 2.53)
pG2A	8.39 (9.54; 7.23)
pG4A	7.78 (12.02; 3.53)
pG5A	8.15 ± 1.72

*SEM is indicated when more than two measures were made.
Otherwise the two measures are given.

[055] The plasmids identified in Table 1 are described in detail hereafter. The following sequence identifiers are used to describe the amino acid and nucleotide sequences of each plasmid insert.

TABLE 2
SEQUENCE IDENTIFIERS

Plasmid Insert	Amino Acid Sequence	Nucleotide Sequence
A	★	★
GA	SEQ ID NO: 1	SEQ ID NO: 7
GlA	SEQ ID NO: 2	SEQ ID NO: 8
G2A	SEQ ID NO: 3	SEQ ID NO: 9
G4A	SEQ ID NO: 4	SEQ ID NO: 10
G5A	SEQ ID NO: 5	SEQ ID NO: 11
SeG5A	SEQ ID NO: 6	12

★The nucleotide sequence of apoaequorin is contained in U.S. 5,422,266.

[056] The identity of the linker used in these constructs is as follows:

DNA sequence of GFP-aequorin linker

pGA (strain I-2507) TCC GGC CTC AGA TCT [SEQ ID NO: 13]

pG1A (strain I-2508) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 14]

pG2A (strain I-2509) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 15]

pG4A (strain I-2510) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 16]

pG5A (strain I-2511) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 17]

pSeG5A (strain I-2512) and pStG5A (strain I-2513) same linker sequence as pG5A.

Peptide sequence of linker

pGA Ser Gly Leu Arg Ser [SEQ ID NO: 18]

pGlA Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 19]

pG2A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 20]

pG4A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 21]

pG5A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 22]

pSeG5A and pStG5A idem than pGSA

[057] Plasmids containing the foregoing polynucleotides have been deposited at the Collection Nationale de Cultures de Microorganismes ("C.N.C.M."), Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France, as follows:

Insert	Plasmid	Accession No.	Deposit Date
A	pAeq+	I-2506	June 22, 2000
GA	pGa	I-2507	June 22, 2000
GlA	pGlA	I-2508	June 22, 2000
G2A	pG2A	I-2509	June 22, 2000
G4A	pG4A	I-2510	June 22, 2000
G5A	pG5A	I-2511	June 22, 2000
SeG5A	pSeG5A	I-2512	June 22, 2000
StG5A	pStG5A	I-2513	June 22, 2000

[058] Recombinant apoaequorin is unstable within the cytosol, with a half-life of approximately 20 minutes (21). In contrast, GFP is a very stable protein and probably stabilizes apoaequorin in the chimeric proteins. The turnover times of the different

cytosolic proteins were estimated on transient expression in COS 7 cells by treatment with puromycin (50μg/ml) for 6 hours. Over this period, most fusion proteins presented a 30% decrease of activity compared with the 80% loss of apoaequorin when alone (Figure 5). It has been observed that, *in vitro*, the fusion proteins of the invention were more sensitive than aequorin alone. G5A gives a significant signal over background with Ca^{++} concentration as low as 38 nM, whereas aequorin needs 28 times more calcium (1μM) to yield a comparable signal (Figure 6). Energy transfer may also improve the quantum yield of GFP-aequorin allowing a more efficient calcium ions detection. To discriminate among the factors contributing to the higher light emission, it will be necessary to study the relaxation mechanisms of the GFP fluorescent excited state on purified hybrid proteins.

[059] More generally, one embodiment of this invention provides a chimeric protein starting with the genes for GFP and aequorin. Improved quantum yield will depend on the functional coupling of the proteins by a Chemiluminescence Resonance Energy Transfer (CRET) mechanism. Thus, after the reconstitution of aequorin and its binding with calcium ions, the activated aequorin transmits its energy to the GFP, which in turn emits a green light to return to its ground state. Optimizing the functional coupling between the two proteins has focused on three points:

1. Improving the induction of a conformational change in the GFP at 37°C, which leads to a higher emission of GFP in the mammalian cells;

2. Changing to the use of aequorin codons adapted to mammalian cells to enhance its expression; and

3. Adding a linkage peptide between the two proteins.

[060] With respect to the third point, an initial molecular construct with five amino acids separating the two proteins was first completed. Then a sequence of nine amino acids was added in a sequence of one to five copies. These constructs were placed in a eukaryote expression vector under control of the CMV (cytomegalovirus) promoter allowing their functional ability to be confirmed. These fusion proteins may be identified: (i) by the GFP signal, through excitation of the biological preparations with light of wavelength 470 nm, by fluorescence microscopy (FITC filter); (ii) by aequorin activity, through emission of blue light after binding with Ca^{++} ions.

[061] The following terms have the following meanings when used herein:

Luminescence

[062] Emission of an electromagnetic radiation from an atom or molecule in UV, in visible or IR. This emission results from the transition from an electronically excited state towards a state from weaker energy, generally the ground state.

Fluorescence

[063] Fluorescence produced by a singlet, very short, excited electronically. This luminescence disappears at the same time as the source from excitation.

Chemiluminescence

[064] Luminescence resulting from a chemical reaction.

Bioluminescence

[065] Visible chemiluminescence, produced by living organisms. The invention mimics the system naturally present in the jellyfish, without fixation to a support.

Bioluminescent system

[066] The bioluminescent system according to the invention is a chimeric tripartite molecule within the middle a peptide linker and a coenzyme (i.e., coelenterazine). The first molecule and the second molecule covalently attached with the linker can be everything if they have for the first a donor site and for the second an acceptor site attached on it (receptors-linker-ligand, antibody-linker antigen). The chimeric protein can be fused to a fragment of tetanus toxin for its retrograde and transynaptic transport on axon by Coen, L., Osta, R., Maury, M., and Brulet, P., Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system. Proc. Natl. Acad. Sci. (USA) 94 (1997) 9400-9405, or fused to a membrane receptor.

Non-radiative

[067] No emission of photon from aequorin to the GTP when aequorin is bounded by calcium ions (therefore there is no transmission of blue light by aequorin in the invention, the energy transfer is directly made between the two proteins).

FRET system

[068] Transfer of energy by resonance by fluorescence (i.e., between two variants of GFP).

References

Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. Nature, (1997) Vol. 388 pp. 882-887.

Detection in living cells of Ca2+-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators.

Romoser, V.A., Hinkle, P.M. and Persechini, A., J. Biol. Chem., (1997) Vol. 272, pp. 13270-13274.

CRET

[069] Transfer of energy by resonance by chemiluminescence (i.e., fusion protein with GFP-aequorin (jellyfish Aequorea) but without linker or GFP-obeline).

Reference:

Chemiluminescence energy transfer.

Campbell, A.K., in Chemiluminescence: Principles and application in Biology and Medicine, Eds Ellis Horwood, Chichester, UK 1988, pp. 475-534.

BRET

[070] Transfer of energy by resonance by bioluminescence (i.e., interaction between GFP and luciferase (jellyfish Renilla).

Reference:

A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock protein.

Xu, Y., Piston, D.W. and Johnson, C.H. Proc. Natl. Acad. Sci., (USA) (1999) Vol. 96, pp. 151-156.

Application 1: Study of calcium signals from a cell population in a eukaryotic organism.

[071] Targeting the bioluminiscent protein sensitive to calcium in a cell population or in a specific tissue may be achieved through homologous recombination or by transgenesis under the control of a specific promoter. Replacing genes by homologous recombination in embryonic cells in mice, such, as *Hoxc-8* and *Otxl,* with this new marker will make it possible to obtain new lines of mutant mice. This approach will permit the detection of electrical activity in a group of neural cells, and will also make it possible to complete the phenotype study of mutants obtained by replacing the *LacZ* gene (Le Mouéllic *et al.*, 1990, 1992; Acampora *et al.*, 1996). For the *Hoxc-8* locus, the expression of the marker should be located in the ventral horns of the spinal chord beginning at section C7 (Le Mouellic *et al.*, 1990). Anomalies in the somatotopic organization of the motor neurons innervating these muscles have been brought to light

(Tiret *et al.*, 1998), and a study of the role of the flux of calcium in the establishment of these neural connections during development may thus be undertaken. In the *Otxl* model, the transgene should be expressed in specific regions of the forebrain, given that an expression localized at layers V and VI of the cerebral cortex, and in regions of the diencephalon, mesencephalon, and cerebellum have been shown (Frantz *et al.*, 1994). Mutant mice obtained by the replacement of the gene by the LacZ gene show a reduction in the thickness of the cerebral cortex and anomalies in the hippocampus, mesencephalon, and cerebellum (Acampora *et al.*, 1996). The loss of balance and rotatory movement observed in these mice can presumably be attributed to anomalies in the sensory organs, specifically in the eye and inner ear. These mice are also subject to generalized epileptic seizures. The establishment of faulty connections and/or abnormal electrical activity could be implicated in the genesis of these pathological processes (McNamara, 1992). The use of this new marker will, on the one hand, make it possible to verify these hypotheses through a functional and dynamic approach, and on the other, to address the development of epilepsy in the adult as well as during development.

Application 2: Study of the role of intraceilular calcium

[072] Calcium is involved in a large number of cellular mechanisms, such as cellular migration, membrane excitability, mitochondrial metabolism, secretion, mitosis, and synaptic plasticity (Berridge *et al.*, 1998). Coding calcium information at the cellular and subcellular level is complex, involving spatial, temporal and quantitative factors. Targeting marker of the invention to different subcellular compartments is possible by fusion with a peptide signal, for example, synoptotagmine.

[073] Example A: Targeting the nuclear compartment will make it possible to study the role of calcium in transcription activation mechanisms and during the mechanisms related to programmed cell death (apoptosis).

[074] Example B: Targeting two fusion proteins with GFP produces different emission spectra in the two cell compartments, for example, cytoplasm and the endoplasmic reticulum will make it possible to study the regulation of the calcium flux during cell activations.

[075] Example C: Targeting the fusion protein in the synapses will make it possible to study the calcium activity linked to electrical activity in neural cells during the release of neurotransmitters. The first possibility is the achievement of a triple fusion between a synaptic protein, such as synaptotagmine or SNAP25, GFP, and aequorin. The existence of protein-protein interactions during exocytosis makes it

possible to consider a second possibility: A functional coupling between GFP and aequorin, the one in fusion with a vesicular protein and the other with a plasma protein. A signal will be obtained only during the interaction of the different proteins in the presence of an increase in the calcium ion concentration.

Application 3: Study of calcium signals at the cell population level

[076] Triple fusing of a protein having intercellular transport properties such as fragment C of the tetanus toxin (TTC) or the VP22 protein of the herpes virus with GFP and aequorin will make it possible to observe the calcium activity in a population of connected cells, for example in a neural network.

Description of the construction of a bioluminescent marker expression vector sensitive to calcium ions

Stage 1: pEGFP-CldKS (*Kpn*I-*Sma*I Deletion)

[077] Double digestion of pEGFP-Cl plasmid (Clontech, see figure) with *Kpn*I and *Sma*I enzymes. After blunt ending the *Kpn*I extension with "Mung bean" nuclease, the two extremities are ligated.

```
5'   GTC GAC GGT ACC GCG GGC CCG GGA TCC 3'
3'   CAG CTG CCA TGG CGC CCG GGC CCT AGG 5'
                 KpnI            SmaI
                  ⇓
     GTC GAC GGT AC            G   GGA TCC
     CAG CTG C                 C   CCT AGG
                  ⇓
     GTC GAC G                 G   GGA TCC
     CAG CTG C                 C   CCT AGG
                  ⇓
          GTC GAC GGG GAT CC
          CAG CTG CCC CTA GG
           SalI          BamHI
```

Stage 2: pEGFP-CImut (GFP mutagenesis)

[078] Four mutagenesis oligonucleotides were used on a single-strand molecule prepared using pEGFP-CIdKS. Each oligonucleotide comprises one or several mismatches (identified below in lower case letters), causing the desired mutation. In the

pEGFP-Clmut plasmid chosen, cut with the *Sac*II enzyme but not the *Age*I enzyme, all of the mutations were verified by sequencing.

- Destruction of the *Age*I site, introduction of a *Sac*II site and deletion of a Valine codon normally absent in "wild-type" GFP (Prasher, D.C., Eckenrode, R.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J., Primary structure of the Aequorea victoria green-fluorescent protein. Gene 111 (1992) 229-233.)

	SacII	Met		Ser	Lys	Gly	Asp	
oGM1 :	5-' GCGCTACCGcggGCCACC	ATG		AGC	AAG	GGC	GAG	3'
pEGFP-CldKS:	5' GCGCTACCGGTCGCCACC	ATG	GTG	AGC	AAG	GGC	GAG	3'
	AgeI		Val					

- Replacement of the 163 Valine codon by an Alanine codon in order to increase the quantity of GFP assuming a correct conformation at 37°C (Siemering, K.R., Golbik, R., Sever, R., and Haseloff, J., Mutations that suppress the thermosensitivity-of green fluorescent protein. Current Biol. 6 (1996) 1653-1663.)

			Ile	Lys	Ala	Asn	Phe	Lys	
oGM2 :	5'	GC	ATC	AAG	Gcc	AAC	TTC	AAG	3'
pEGFP-CldKS	5'	GC	ATC	AAG	GTG	AAG	TTC	AAG	3'
					Val				

- Replacement a 231 Leu codon by a Histidine codon normally present in "wild-type" GFP (Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J., Primary structure of the Aequorea victoria green-fluorescent protein. Gene 111 (1992) 229-233.)

			Ile	Thr	His	Asn	Met		
oGM3 :	5'	GG	ACT	ATC	CaC	GGC	ATG	GA	3'
pEGFP-CldKS :	5'	GG	ACT	ATC	CTC	GGC	ATC	GA	3'
					Leu				

Stage 3: pEGFPmut-Aeq (GFP-Aeguorin fusion protein)

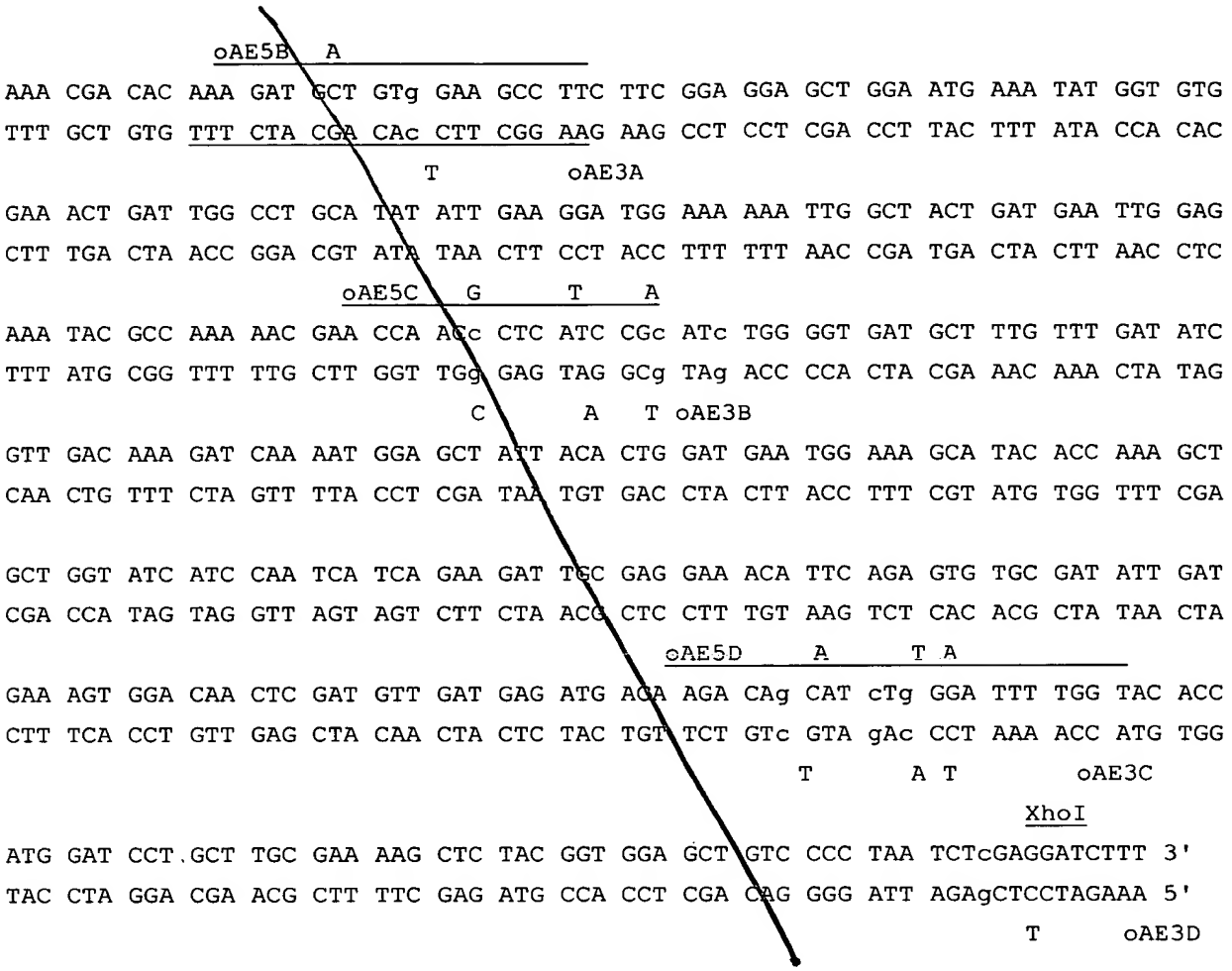
[079] Four PCRs (Polymerase Chain Reaction) done on a vector comprising the aequorin (Aeq) coding phase makes it possible to amplify the A, B, C, and D fragments with, respectively, the primers oAE5A and oAE3A, oAE5B and oAE3B, oAE5C and oAE3C, oAE5D and oAE3D. The overlapping regions are used to assemble the

different parts during successive PCRs (Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. Site-directed mutagenesis by overlap extension using the polymerase chain reaction Gene 77 (1989) 51-59.) An A+B fragment is amplified starting with a mixture of A and B fragments, and the primers oAE5A and oAE3B. Similarly, a C+D fragment is amplified with a mixture of C and D fragments, using the primers oAESA and oAE3D. Finally, the complete coding phase, A+B+C+D is developed with the primers oAE5A and oAE3D.

- Each oligonucleotide comprises one or several mismatches that are identified below in lower case. The "wild" sequence is represented opposite, in upper case. The primer oAE5A suppresses the original initiation translation code (ATG) and introduces a *Bgl*II site. The primer oAE3D introduces an *Xho*I site just behind the translation terminal codon (TAA). The final PCR product, digested with the *Bgl*II and *Xho*I enzymes, is cloned in the *Bgl*TI-*Sal*I sites of the pEGFP-Clmut plasmid in such a way that the Valine codon (GTC), the first codon of aequorin, is in the same reading phase as the GFP (see figure). The other primers introduce "silent" mutations that do not change the protein sequence but modify six codons in the jellyfish, *Aequoria victoria*, to improve their expression in mammals (Wada, K.-N., Aota, S.-I., Tsuchiya, R., Ishibashi, F., Gojobori, T., and Ikemura, T. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. 18 suppl. (1990) 2367-2411.). The completeness of the entire sequence was verified by sequencing,

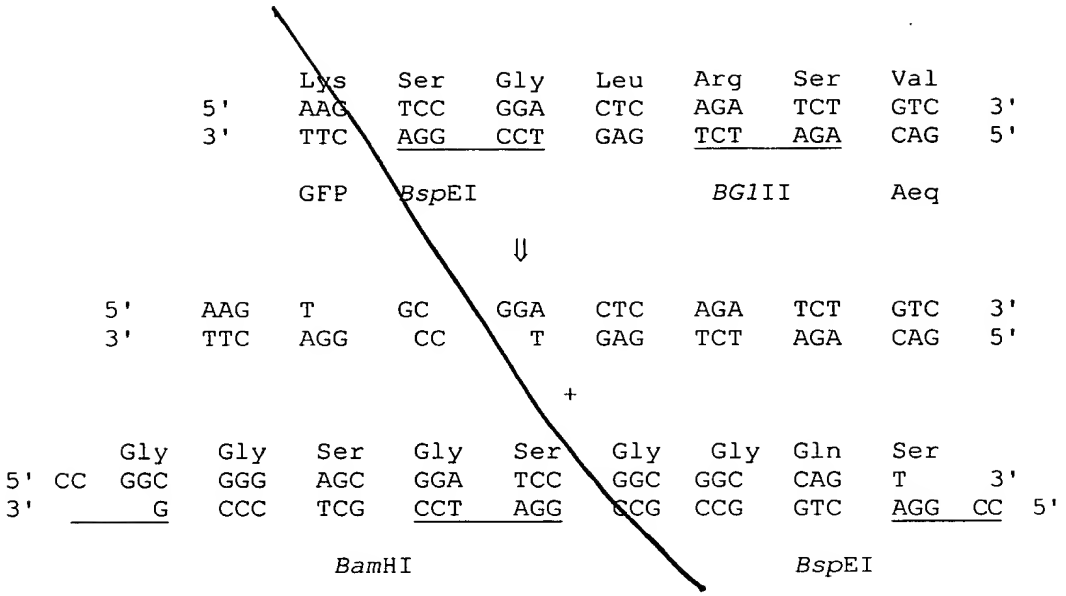
```
      oAE5A   CCATG
5'    AGCTTCAgatct GTC AAA CTT ACA TCA GAC TTC GAC AAC CCA AGA TGG ATT GGA CGA
3'    TCGAAGTctaca CAG TTT GAA TGT AGT CTG AAG CTG TTG GGT TCT ACC TAA CCT GCT
           BGlII

CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG
GTG TTC GTA TAC AAG TTA AAG GAA CTA CAG TTG GTG TTA CCT TTT TAG AGA GAA CTG CTC
ATG GTC TAC AAG GCA TCT GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC
TAC CAG ATG TTC CGT AGA CTA TAA CAG TAG TTA TTG GAA CCT CGT TGT GGA CTC GTT CGG
```



Stage 4: pGCA (Insertion of an *Intercalated* sequence)

[080] In the pEGFPmur-Aeq plasmid, a sequence of five amino acids exists between the coding phases of the GFP and aequorin. Observations led to the lengthening of this region by intercalating a sequence in the *Bsp*EL site. Two complementary oligonucleotides coding for a sequence of nine amino acids give the composition a good deal of flexibility, owing to the abundance of Glycine and Serine. After insertion, the *Bsp*EI site is preserved on only one side although new intercalated sequences may be added successively. At each stage, the orientation is controlled by the *Bsp*EI enzyme. Two copies of this sequence are needed to restore the normal fluorescence of GFP, but the energy transfer between aequorin and GFP is optimal with five copies. The entire intercalated sequence of pGCA plasmid (5 x 9 aa + the five initial amino acids = 50 aa) was verified by sequencing:



[081] Optimization of the energy transfer by inserting a spacer between GFP and Apoaequorin.

[082] A non-radiative energy transfer between the excited oxyluciferin and the GFP chromophore will be strongly dependent upon their overall geometry and their respective motions. Therefore, a linker was designed principally composed of serine and glycine residues to intercalate a flexible element of variable length.

[083] The ratio of green and blue photons emitted upon Ca^{++} triggering has been measured on cellular extracts prepared 48h after transient transfection of Neuro2A cells. The photons emitted through a beam-splitter were counted after passing appropriate filters. Covalent linking of GFP to aequorin (GA) significantly modified the wavelength of maximum light emission (Fig. 2), thereby demonstrating intramoiecular energy transfer. The ratio of green over blue light (500/450nm) was further raised from 3 to around 7 by adding 1 to 5 linkers (Fig. 2, CRET). Preliminary measurement indicates that this ratio can reach almost 11 with SG5A probably because of the accumulation of the fusion protein anchored to the membranes (see materials and methods).

[084] Spectral emissions of the different constructs were also analyzed using a monochromator. Aequorin showed a broad spectrum with maximum wavelength at 474 ± 6.9 nm and a bandwidth, corresponding to the distance between low and high wavelengths at 50% values of the maximum emission, at 108.3 ± 20.1 nm (Fig. 2). There was a clear shift toward the green in the peak emission of the GFP-aequorin constructions ranging from 506.7 ± 1.2 nm to 514.1 + 3.4 nm. Increasing the length of

the linker further affected the sharpness of the spectrum, as indicated by the narrower bandwidths, 88.4 + 9.4 nm and 56.0 $\pm$ 3.3 nm, for pGA and pG5A respectively. There was no evidence of a bimodal spectrum with any of the pG1A-pG5A constructs indicating an optimal transfer which could be incomplete in the case of pGA.

[085] When the spacer between GFP and aequorin is longer than 14 amino acids, the donor and the acceptor dipoles have probably more freedom to be in a configuration favourable for optimum intramolecular energy transfer. The system of the invention yields an efficiency comparable to the intermolecular CRET measured *in vivo* (22, 23) and provides a convenient model for the biophysical studies of radiationless energy transfer mechanisms.

[086] Cellular localization and targeting of GFP-Apoaequorin.

[087] The cellular localization of the GFP-apoaequorin constructs has been examined. Figure 3 illustrates GFP activity 48h after transient transfection in Neuro2A cells. Expression of the mutant GFP alone (Gm) showed homogenous fluorescence in the cytosol as well as in the nucleus as expected since GFP is a small protein that can diffuse into the nucleus. Mutation V163A improves remarkably the fluorescence signal and reduces photobleaching when compared to the original EGFP (data not shown) probably owing to a higher concentration of properly folded protein. An evenly distribution is also observed for all the GFP-apoaequorin constructions in Neuro2A cells (Fig. 3A-D) as well as in COS-7 cells. Bright spots often appeared in the cytosol with fusion proteins having the shortest linkers: GA, GlA and G2A. These spots were less frequent with G4A and never observed with Gm and G5A. High concentrations of proteins expressed during transient transfections could induce the aggregation of GFP (24), which is also going to be influenced by the presence of the aequorin protein and the distance separating them.

[088] The GFP-apoaequorin has also been targeted to the neurotransmitter vesicles with a complete or a partial synaptotagmin I molecule. Synaptotagmin I is a transmembrane protein of synaptic vesicles and is implicated in neurotransmitter exocytosis (25). For imaging calcium microdomains in presynaptic compartments, the signal should be more accurate than if evenly distributed in the cytoplasm of neurons. In a three part fusion protein, SG5A (Fig. 1), the complete coding sequence of synaptotagmin I has been put in frame upstream of G5A. In this case, GFP fluorescence is superimposable with synaptotagmin immunostaining but is also visible at the cellular surface (Fig. 3E). In neurons (26) and in Neuro2A cells, synaptotagmin I is localized in

neuronal processes, but is undetectable in plasma membranes, probably because the dynamic mechanisms of exocytosis are followed by rapid endocytosis. When GFP-apoaequorin is fused with only the N-terminal part of synaptotagmin including the transmembrane domain but lacking the cytoplasmic domain (tSG5A, Fig. 1), a strong fluorescence is restricted to the cytosol (Fig. 3F). The punctate labeling suggests that this protein is locked into the trans-golgi system. The correct targeting of the three part fusion molecule of the invention does not occur with tSG5A and appears to be slowed down in the case of SG5A. When fused to the complete synaptotagmin protein, the bioluminescent marker is held back in the plasma membrane, but nevertheless labels all neurite outgrowths present in Neuro2A cells.

[089] Ca^{++} detection in single cells.

[090] Neuro2A cells were transiently transfected with pA, pGA, pG2A, pG5A or cotransfected with pA and pGm (Fig. 1). After aequorin reconstitution with native coelenterazine in Ca^{++} - free buffer, an emission of photons has been measured with a classical intensified CCD camera upon the addition of CaCl2 solution (5 mM) (Fig.4A.1 and 4A.4). With the negligible background (Fig. 4A.2), integration time of 1 second is enough to record the signal in single cells (Fig.4A.1) expressing any of the fusion proteins. No signal could be visualized with aequorin alone or with co-expressed free GFP (data not shown). The presence of unbound GFP does not improve aequorin chemiluminescence as we observed *in vitro*. Because of the low level of light produced, aequorin expressed *in situ* has never been detected in single cells except when targeted in mitochondria. With a cooled intensified CCD camera, *Rutter et al.* (1996) (27) have succeeded in detecting intramitochondrial Ca^{++} signals when aequorin is fused to cytochrome *c* oxidate. Transgenes encoding cytoplasmic aequorin can report calcium activities in monolayers of cells only when photomultipliers (PMT) are used, which are more sensitive but lack the spatial resolution for single cell analysis. The stability of GFP-aequorin fusions of the invention and the improved light emission have made it possible to detect physiological Ca^{++} signals at the level of single cells.

[091] Calcium deficiency prior to measurements or the transfection conditions used may induce cellular depolarization, such that opening of the voltage dependent Ca^{++} channels is likely to be responsible for the fast bioluminescent response to $CaCl_2$, addition (Fig. 4A). Light emission would then return to background level because of the desensitization of Ca^{++} channels and the membrane depolarization by Ca^{++}-dependent K' channels (28). Fluo-3 showed a similar profile in mock transfections of Neuro2A cells

(Fig. 4C). Subsequent addition of a Ca^{++} ionophore (A23187) induced a second emission of photons with comparable intensity but with different kinetics. A lower light intensity is detectable in Neuro2A cells transfected with pSG5A (Fig. 4B). When a fluorescent calcium probe is anchored to the inner surface of the membrane, the response kinetics are much quicker than when the probe is not targeted (29). The use of the bioluminescent reporter SG5A probably requires a system with higher spatial and temporal resolutions. In any case, the responses observed are not due to the complete consumption of aequorin as more bioluminescence can still be observed when a concentrated Ca^{++} solution (100mM) is applied to cells (see Fig. 4B for example). For each construction, measurements have been repeated at least 4 times. A variability of individual cells responses was observed, probably due to cell population heterogeneity. Further investigations are required to calibrate relative light unit (RLU) versus Ca^{++} concentrations. Patch-clamp techniques will also allow the identification of the type of calcium channels implicated in these responses and the effect of cellular transfection on membrane potential.

[092] The transgenes of the invention should permit imaging of electrical activity in neural networks in whole animals. *In vitro*, two approaches were used until recently. The first method is based on the coupling of exocytosis to emission of light from synaptolucins in nerve cells (30). Light emission occurs when the luciferase, targeted inside the synaptic vesicles, reacts with ATP in the extracellular space. With this system, the authors obtain signals correlated with the neurotransmitter release but the low light level requires very long acquisition times (over 30 sec). In the second approach, fluorescence Ca^{++} sensitive markers have been used for measurements of intracellular [Ca^{++}] by FRET (3, 4, 31). For single cell detection, this technique requires a sufficient concentration of probe to discriminate the signal from the background which is generated by autofluorescence of biological compounds and the possibility of calcium-independent energy transfer between the two GFPs. The integration times are also relatively long, between 4 and 20 seconds.

[093] This invention thus provides new bifunctional hybrids in which expression patterns can be followed by GFP fluorescence while the aequorin moiety is the reporter of Ca^{++} activity. Furthermore, the functional coupling of the two components, which follows the CRET principle, results in a higher amount of light emission and a greater Ca^{++} sensitivity. Bioluminescent activities of these genetic markers have been assessed in single cells with a cooled intensified CCD camera in 1

second integration times. The recent development of low level light detection systems should allow detection of CRET signals with much shorter integration times and higher spatial resolution. Intracellular and intercellular Ca^{++} signaling can be approached *in vivo* in transgenic animals in which the GFP-aequorin is targeted to a particular cell population and/or to specific subcellular compartments. Particularly, calcium oscillations can then be imaged simultaneously in cells of an integrated neural circuitry in real time.

[094] This invention will be described in greater detail in the following Examples.

EXAMPLE 1

Construction of GFP-aequorin fusion proteins

[095] All the constructs were made in the pEGFP-Cl vector (Clontech). The EGFP gene is codon-optimized for maximal expression in mammalian cells. It also contains 2 mutations in the chromophore, F64L and S65T, which modify the excitation spectra and enhance fluorescence intensity (17). We further substituted valine 163 of the EGFP by alanine, using single strand mutagenesis, to improve the proper folding of the protein and increase the fluorescence at 371C (18, 19). The aequorin coding sequence, a generous gift by M.-T. Nicolas, has been fused in frame at the 3' end of the EGFP gene in the BgIII/SaII sites of pEGFP-Cl. Seven codons were modified for a better expression in mammalian cells by means of site-directed mutagenesis using PCR (polymerase chain reaction) with overlap extension. Then, complementary oligonucleotides, 5'-CCGGCGGGAGCGGATCCGGCGGCCAGT-3' [SEQ ID NO: 23] and 5'-CCGGACTGGCCGCCGGATCCGCTCCCG-3' [SEQ ID NO: 24] were inserted at the *Bsp*EI site in the 15 bp sequence between GFP and aequorin. Conservation of the *Bsp*EI site at only one end allowed sequential addition of one to five linker sequences (pG1A-pG5A).

[096] Two additional fusion constructs were made in pG5A with a synaptic protein, synaptotagmin I of which the cDNA plasmid was generously gift by M. Fukuda. Sequences encoding for either the entire open reading frame or the first 134 N-terminal amino acids, comprising the transmembrane domain of the protein, were fused in frame at the 51 end of the GFP-aequorin gene.

EXAMPLE 2

Cell Culture and transfection

[097] Neuroblastoma cells (Neuro2A, mouse) were grown in Dulbecco's Eagle medium (Life Technologies - Gibco, UK) supplement with 10% (V/V) heat-treated foetal calf serum, 2mm glutamine (Life Technologies - Gibco, UK) and 100 units streptomycin-penicillin (Life Technologies - Gibco, UK). The culture were incubated at 370C in a humidified atmosphere containing 8t C02 and transiently transected using either the $CaPO_4$, technique or the FuGENE 6™ transfection reagent (Roche).

EXAMPLE 3

In vitro Ca++ sensitive chemiluminescence and CRET activities

[098] Cells were harvested 48h after transfection in 250µl of 10mM β-mercaptoethanol, 4mM EDTA, 5μM coelenterazine in PBS at 4°C during 2 to 4 hours. Cells were rinsed in lmM EDTA in PBS and harvested in 400μl of hypo-osmotic buffer (20mM Tris-HC1 pH 7.5/ 5mM EDTA/ 5mM β-mercaptoethanol with a protease inhibitor cocktail according to the manufacturer, Roche), for 30min. to 1h. at 4°C. The cell membrane were broken by passing through a 30 gauge needle and the cellular extract was obtained after microcentrifugation at 13000 rpm for lh at 40C. The supernatant was harvested for all constructions but SGSA for which the membrane pellet was further resuspended. Calcium sensitivity chemiluminescent activity was measured in a luminometer (Lumat LB95501 E&EG Berthold). Aliquots (10µl) were placed in sample tube (with 90µl of 10mM Tris-HCl pH 7.5) in the luminometer and the light intensity expressed in relative light unit (R.L.U.) was measured after the injection of 100µl of 50mM $CaCl_2$/10mM Tris-HC1 pH 7.5 solution.

[099] For CRET measurements, aliquots of extracts from transfected cells were placed in a reservoir chamber and brought into contact with an optic fibre bundle attached to a photon counting camera (Photek three-microchannel plate intensified CCD camera: Photek 216). Before capture of signals, light passes through a monochromator allowing the spectral analysis of emitted photons. The acquisition begins 20 seconds before injection of $CaCl_2$ and carries on during 40 seconds after injection of the $CaCl_2$ solution (50mM). For green/blue photons ratio determinations, the same procedure was followed but in this case the system measures the light emitted through blue (450nm) and green (500nm) filters after a beam splitter.

EXAMPLE 4

GFP fluorescence and immunolocalization

[0100] Neuro2A cells were fixed 48h after transfection in 4% paraformaldehyde in PBS pH 7.4, rinsed in PBS, and mounted. GFP fluorescence is visualized under a confocal Laser Scanning microscope (Zeiss, Heidelberg, Germany) which uses an argon-krypton laser operating in multi-line mode or an Axiophot microscope with an epiluminescent system (Zeiss, Heidelberg, Germany). For immunolocalisation of the targeted GFP-aequorin, fixed cells were pre-treated with 50mM NH_4Cl in PBS pH 7.4 for 5 min. at room temperature, permeabilised in 2% BSA/ 0.02% Triton/goat serum solution in PBS during lh. Antibodies against synaptotagmin (StressGen SYA-130) were then applied during 2-4 hrs. Cells were then rinsed in PBS and incubated in 2% BSA/ 0.02% Triton in PBS with secondary antibody diluted at 1/100 (TRITC conjugated antibody). Cells were then washed in PBS and mounted.

EXAMPLE 5

Single cells bioluminescence detection

[0101] Forty-eight hours after transfection, cells were rinsed in 124mM NaCl/ 5mM KCl/ 15mM Hepes pH 7.4/ 5mM $NaHCO_3$/ 1mM NaH_2PO_4/ 0.5mM $MgSO_4$/ 1. 5mM $CaCl_2$ / 5.5 mM Glucose and later incubated in the same buffer without $CaC1_2$ with 5μM coelenterazine to reconstituted aequorin, for 2 to 4h at 37°C and then rinsed. Calcium signals were visualized with a modified Olympus upright microscope (BHS) fitted with an BH2-RFCA epifluorescence unit recorded through a plan x40 Olympus long working distance water-immersion lens (N.A. 0.7). GFP Fluorescence allowed to choose the recording area on transfected cells. The excitation lamp was shut off and the gain of the camera increased. Images were integrated every second with a cooled Photonic Science extended ISIS video camera. Each profile in Figure 4 represents the amount of light emitted over the area that we defined around the soma of individual cells using the Axon Imaging Workbench 2214 software. Intensities of fluorescence and CRET activity are translated in scaled pseudocolors. Controls were made with Fluo-3 AM on mock-transfected Neuro2A cells to check the experimental conditions.

EXAMPLE 6

Protein stability

[0102] The turnover times of the different cytosolic proteins were estimated on transient expression in COS7 cells by treatment with puromycin (50μg/ml) for 6h. Ca^{2+}-induced chemiluminescence activities were performed on cellular extract obtained

after the reconstitution of aequorin in presence of 5μM coelenterazine. Calcium sensitivity chemiluminescence activity was measured in a luminometer (Lumat LB95501 E&EG Berthold). Aliquots (10μl) were placed in a sample tube (with 90μl of 10mM Tris-HCl, pH 7.5) in the luminometer and the light intensity expressed, in relative light units (RLUs), was measured after the injection of 100μl of 50mM $CaCl_2$/10mM Tris-Hcl pH 7.5 solution. Relative chemiluminescence activities are expressed as a percentage of the activity at the time zero (100%). The results are shown in Fig. 5. As seen in Fig. 5, over this period, most fusion proteins presented 30% decrease of activity compared with the 80% loss of apoaequorin when alone.

EXAMPLE 7

Determination of the Ca++ affinity of aequorin and G5A

[0103] Ca^{2+}- induced chemiluminescence activities were performed on cellular extract obtained after the reconstitution of aequorin in presence of 5μM coelenterazine. Calcium sensitivity chemiluminescence activity was measured in a luminometer (Lumat LP95501 E&EG Berthold). Aliquots (10μl) were placed in a sample tube (with 90μl of 10mM Tric-HC1, pH 7.5) in the luminometer and the light intensity expressed, in relative light units (RLUs), was measured after the injection of 100μl of different Ca/EGTA solutions. The results are shown in Fig. 6. As seen in Fig. 6, G5A gives a significant signal over background with Ca^{2+} concentrations as low as 38 nM, whereas aequorin needs 28 times more calcium (1 M) to yield a comparable signal.

For **Chimeric GFP-aequorin as bioluminescent Ca^{2+} reporters at the single cell level**

Concerning the invention of chimeric GFP-aequorin calcium sensitive bioluminescent reporters, we have developed new applications and we have some preliminary datas about sensitivity of GFP-aequorin proteins to Ca^{2+} ions.

EXAMPLE 8

Ca^{2+} sensitivity of G5A and SG5A: Calibration curves between bioluminescent signals and Ca^{2+} concentrations

Measurements of Ca^{2+} sensitivity of two constructs G5A and SG5A were performed on cellular extracts obtained after the reconstitution of aequorin in presence of 5μM colenterazine. Calcium chemiluminescence activity was measured in a luminometer (Lumat LB95501 E&EG Berthold). Aliquots (10μl) were placed in a

sample tube with 90 µl of 10mM Tris.HCl pH 7.5 in the luminometer and the light intensity expressed, in relative light units (RLUs), was measured after the injection of 100ml of different Ca/EGTA solutions (Molecular Probes Calcium Calibration Buffer Kit). Figure 7 shows the Ca^{2+} response curve of G5A, SG5A and aequorin. The curves represent the relationship between the ratio L/Lmax and [Ca2+]. L is the rate of RLUs at any given [Ca2+] and Lmax is the rate of RLUs at saturating [Ca2+]. These results show a much higher affinity for Ca^{2+} of the various forms of GFP-aequorin than aequorin.

EXAMPLE 9

New applications of GFP-aequorin reporters

Adenoviral vectors with GFP-aequorin were developed. Using these new constructs, dissociated neurons from rat spinal cord in culture can be transfected with higher efficiency. Figures 8 and 9 depict Ca^{2+}-induced bioluminescent signals detected at the single cell level in dissociated neuronal cells. Neuronal cells infected by adenoviral vectors with G5A (Fig. 8) or SG5A (Fig. 9) were pre-incubated with 5µM coelenterazine in a Ca^{2+}-free buffer. Intensities of fluorescence and bioluminescence activity are translated in pseudocolors. Representative pictures of the chosen fields are shown after the addition of 5mM and 2.5mM of $CaCl_2$, respectively, for Figures 8a-c & 9a at 12 and 9 seconds. Figures 8d-e and 9b were obtained after addition of ionomycin and high concentration of $CaCl_2$ (100mM).

EXAMPLE 10

Expression of GFP-aequorin reporters *in vivo* in *Xenopus* embryos and measurement of calcium activities

Calcium signalling during early and late embryogenesis in *Xenopus* was studied. Figure 10 shows representative pattern of luminescence activity illustrating the changes in intracellular calcium during the neural induction after the injection of the GA plasmid at the one cell stage in *Xenopus* embryo. Figure 11 shows a transgenic *Xenopus* larva with GFP-aequorin. These techniques can also be employed with zebrafish and mouse transgenics. These results show that these calcium reporters can be used in a great variety of organisms or tissues to visualize calcium activity and to measure calcium concentrations.

[0104] In summary, the new linker useful for energy transfer by CRET system in a bioluminescent system has the following properties:

Forms:

[0105] Different amino acid sequences and peptide sequences of the linker are described. Its length comprises a minimal size of 4 to 9 amino acids, which can be extended by a group of 7 to 12 amino acids (in a preferred embodiment 9 amino acids). The said group is extendable to 63 amino acids, i.e., 9 x 6 times. The experiment was done, for example, with a peptide linker comprising 5 amino acids followed by 1 to 5 times of 9 amino acids.

Functions:

[0106] Its first function is to approach donor sites and acceptor sites of two molecules for a direct transmission of energy. This linker confers an optimal environment for energy transmission by CRET.

[0107] The second function is the stabilization of the described system by increasing the half life of aequorin because of the fusion of GFP. The aequorin is linked to the GFP, which has a half life of more than 24 hours.

Applications:

[0108] In a bioluminescent system, aptitude for protein-protein interaction.

[0109] Application of the bioluminescent system with the linker: epileptogenesis, SNC disease (visualization of the neuronal cell activities during development and in the adult), neuromuscular connection with the implication of homeogene HOX-C8 in the spinal cord.

[0110] Application in apoptosis with a chimeric protein comprising the linker according to the invention by the visualization of the modifications of the intracellular calcium pools.

[0111] Visualization and precision of the role of calcium waves in living organs like the spleen (intra and intercellular calcium waves).

Results:

[0112] Chimeric protein is more stable by augmentation of the half-life of the molecule. Augmentation of the sensitivity for calcium ions is important.

[0113] The linker of the invention has surprising properties. The sensitivity of calcium ions of the chimeric molecule containing the aequorin and the linker is different from that for aequorin alone. The invention provides a better sensitivity.

[0114] This linker makes it possible to attach together an aequorin molecule with a GFP. The following reference demonstrates that the both molecules do not interact together without a linker: Morise, H. Shimomura, O., Johonson, F.H. and Winant, J.

(1974) Intermolecular Energy Transfer in the bioluminescent system of Aequoria. Biochemistry 13, 2656-2662.

[0115] It is the first time that one can obtain visualization of aequorin signal in a live single cell system (or in an alive animal).

[0116] In summary, monitoring calcium fluxes in real time could help to understand the development, the plasticity and the functioning of the central nervous system. In jellyfish, the chemiluminescent calcium binding aequorin protein is associated with the green fluorescent protein (GFP) and a green bioluminescent signal is emitted upon Ca^{++} stimulation. We decided to use this Chemiluminescence Resonance Energy Transfer (CRET) between the two molecules. Calcium sensitive bioluminescent reporter genes have been constructed by fusing GFP and aequorin resulting in much more light being emitted. Chemiluminescent and fluorescent activities of these fusion proteins have been assessed in mammalian cells. Cystosolic Ca^{++} increases were imaged at the single cell level with a cooled intensified CCD camera. This bifunctional reporter gene should allow the investigation of calcium activities in neuronal networks and in specific subcellular compartments in transgenic animals.

[0117] Following are sequences and the corresponding sequence identifiers referred to herein:

Peptide sequences:

GA

M S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T
Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D
H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F
E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I
M A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D
G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G
I T H G M D E L Y K **S G L R S** V K L T S D F D N P R W I G R H K H M F N F L
D V N H N G K I S L D E M V Y K A S D I V I N N L G A T P E Q A K R H K D A
V E k F F G G A G M K Y G V E T D W P A Y I E G W K K L A T D E L E K Y A K
N E P T L I R I W G D A L F D I V D K D Q N G A I T L D E W K A Y T K A A G
I I Q S S E D C E E T F R V C D I D E S G Q L D V D E M T R Q H L G F W Y T
M D P A C E K L Y G G A V P [SEQ ID NO: 1]

G1A

M S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T
Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D
H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F
E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I
M A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D
G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G
I T H G M D E L Y K **S G G S G S G G Q S G L R S** V K L T S D F D N P R W I G
R H K H M F N F L D V N H N G K I S L D E M V Y K A S D I V I N N L G A T P
E Q A K R H K D A V E A F F G G A G M K Y G V E T D W P A Y I E G W K K L A
T D E L E K Y A K N E P T L I R I W G D A L F D I V D K D Q N G A I T L D E
W K A Y T K A A G I I Q S S E D C E E T F R V C D I D E S G Q L D V D E M T

R Q H L G F W Y T M D P A C E K L Y G G A V P [SEQ ID NO: 2]

G2A

M S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T
Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D
H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F
E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I
M A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D
G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G
I T H G M D E L Y K **S G G S G S G G Q S G G S G S G G Q S G L R S** V K L T S
D F D N P R W I G R H K H M F N F L D V N H N G K I S L D E M V Y K A S D I
V I N N L G A T P E Q A K R H K D A V E A F F G G A G M K Y G V E T D W P A
Y I E G W K K L A T D E L E K Y A K N E P T L I R I W G D A L F D I V D K D
Q N G A I T L D E W K A Y T K A A G I I Q S S E D C E E T F R V C D I D E S
G Q L D V D E M T R Q H L G F W Y T M D P A C E K L Y G G A V P
[SEQ ID NO: 3]

G4A

M S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T
Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D
H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F
E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I
M A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D
G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G
I T H G M D E L Y K **S G G S G S G G Q S G G S G S G G Q S G G S G S G G Q S**
G G S G S G G Q S G L R S V K L T S D F D N P R W I G R H K H M F N F L D V
N H N G K I S L D E M V Y K A S D I V I N N L G A T P E Q A K R H K D A V E
A F F G G A G M K Y G V E T D W P A Y I E G W K K L A T D E L E K Y A K N E
P T L I R I W G D A L F D I V D K D Q N G A I T L D E W K A Y T K A A G I I
Q S S E D C E E T F R V C D I D E S G Q L D V D E M T R Q H L G F W Y T M D
P A C E K L Y G G A V P [SEQ ID NO: 4]

G5A

M S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T
Y G K L T L K F I C T T G K L P V P W P T L V T T L T Y G V Q C F S R Y P D
H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F
E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I
M A D K Q K N G I K A N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D
G P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G
I T H G M D E L Y K **S G G S G S G G Q S G G S G S G G Q S G G S G S G G Q S**
G G S G S G G Q S G G S G S G G Q S G L R S V K L T S D F D N P R W I G R H
K H M F N F L D V N H N G K I S L D E M V Y K A S D I V I N N L G A T P E Q
A K R H K D A V E A F F G G A G M K Y G V E T D W P A Y I E G W K K L A T D
E L E K Y A K N E P T L I R I W G D A L F D I V D K D Q N G A I T L D E W K
A Y T K A A G I I Q S S E D C E E T F R V C D I D E S G Q L D V D E M T R Q
H L G F W Y T M D P A C E K L Y G G A V P [SEQ ID NO: 5]

SeG5A

M V S A S R P E A L A A P V T T V A T L V P H N A T E P A S P G E G K E D A
F S K L K Q K F M N E L H K I P L P P W A L I A I A I V A V L L V V T C C F
C V C K K C L F K K K N K K K G K E K G G K N A I N M K D V K D L G K T M K
D Q A L K D D D A E T G L T D G E E K E E P K E E E K L G K L Q Y S L D Y D
F Q N N Q L L V G I I Q A A E L P A L D M G G T S D P Y V K V F L L P D K K
K K F E T K V H R K T L N P V F N E Q F T F K V P Y S E L G G K T L V M A V
Y D F D R F S K H D I I G E F K V P M N T V D F G H V T E E W R D L Q S A E
K E E Q E K L G D I C F S L R Y V P T A G K L T V V I L E A K N L K K M D V
G G L S D P Y V K I H L M Q N G K R L K K K K T T I K K N T L N P Y Y N E S
F S F E V P F E Q I Q K V Q V V V T V L D Y D K I G K N D A I G K V F V G Y
N S T G A E L R H W S D M L A N P R R P I A Q W H T L Q V E E E V D A M L A
V K R S G N S G R A T M S K G E E L F T G V V P I L V E L D G D V N G H K F
S V S G E G E G D A T Y G K L T L K F I C T T G K L p V P W P T L V T T L T

Y G V Q C F S R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D
G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L
E Y N Y N S H N V Y I M A D K Q K N G I K A N F K I R H N I E D G S V Q L A
D H Y Q Q N T P I G D G P V L L P D N H Y L S T Q S A L S K D P N E K R D H
M V L L E F V T A A G I T H G M D E L Y K **S G G S G S G G Q S G G S G S G G**
Q S G G S G S G G Q S G G S G S G G Q S G G S G S G G Q S G L R S V K L T S
D F D N P R W I G R H K H M F N F L D V N H N G K I S L D E M V Y K A S D I
V I N N L G A T P E Q A K R H K D A V E A F F G G A G M K Y G V E T D W P A
Y I E G W K K L A T D E L E K Y A K N E P T L I R I W G D A L F D I V D K D
Q N G A I T L D E W K A Y T K A A G I I Q S S E D C E E T F R V C D I D E S
G Q L D V D E M T R Q H L G F W Y T M D P A C E K L Y G G A V P
[SEQ ID NO: 6)

GA

Atg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac
ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc
tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg
ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac
cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc
gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac
ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc
atg gcc gac aag cag aag aac ggc atc aag gCC aac ttc aag atc cgc cac aac atc
gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa
gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg
atc act cAc ggc atg gac gag ctg tac aag **tcc gga ctc aGA TCT** gtc aaa ctt aca
tca gac ttc gac aac cca aga tgg att gga cga cac aag cat atg ttc aat ttc ctt
gat gtc aac cac aat gga aaa atc tct ctt gac gag atg gtc tac aag gca tct gat
att gtc atc aat aac ctt gga gca aca cct gag caa gcc aaa cga cac aaa gat gct
gtG gaa gcc ttc ttc gga gga gct gga atg aaa tat ggt gtg gaa act gat tgg cct
gca tat att gaa gga tgg aaa aaa ttg gct act gat gaa ttg gag aaa tac gcc aaa
aac gaa cca acC ctc atc cgC ata tgg ggt gat gct ttg ttt gat atc gtt gac aaa
gat caa aat gga gct att aca ctg gat gaa tgg aaa gca tac acc aaa gct gct ggt
atc atc caa tca tca gaa gat tgc gag gaa aca ttc aga gtg tgc gat att gat gaa
agt gga caa ctc gat gtt gat gag atg aca aga caG cat CtG gga ttt tgg tac acc
atg gat cct gct tgc gaa aag ctc tac ggt gga gct gtc ccc
[SEQ ID NO: 7]

GlA

Atg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac
ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc
tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg
ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac
cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc
gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac
ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc
atg gcc gac aag cag aag aac ggc atc aag gCC aac ttc aag atc cgc cac aac atc
gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa
gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg
atc act cAc ggc atg gac gag ctg tac aag **tcc ggc ggg agc gga tcc ggc ggc cag**
tcc ggc ctc aGA TCT gtc aaa ctt aca tca gac ttc gac aac cca aga tgg att gga
cga cac aag cat atg ttc aat ttc ctt gat gtc aac cac aat gga aaa atc tct ctt
gac gag atg gtc tac aag gca tct gat att gtc atc aat aac ctt gga gca aca cct
gag caa gcc aaa cga cac aaa gat gct gtG gaa gcc ttc ttc gga gga gct gga atg
aaa tat ggt gtg gaa act gat tgg cct gca tat att gaa gga tgg aaa aaa ttg gct
act gat gaa ttg gag aaa tac gcc aaa aac gaa cca acC ctc atc cgC ata tgg ggt
gat gct ttg ttt gat atc gtt gac aaa gat caa aat gga gct att aca ctg gat gaa
tgg aaa gca tac acc aaa gct gct ggt atc atc caa tca tca gaa gat tgc gag gaa

aca ttc aga gtg tgc gat att gat gaa agt gga caa ctc gat gtt gat gag atg aca
aga caG cat CtG gga ttt tgg tac acc atg gat cct gct tgc gaa aag ctc tac ggt
gga gct gtc ccc [SEQ ID NO: 8]

G2A

Atg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac
ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc
tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg
ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac
cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc
gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac
ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc
atg gcc gac aag cag aag aac ggc atc aag gCC aac ttc aag atc cgc cac aac atc
gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa
gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg
atc act cAc ggc atg gac gag ctg tac aag **tcc ggc ggg agc gga tcc ggc ggc cag**
tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ctc aGA TCT gtc aaa ctt aca tca
gac ttc gac aac cca aga tgg att gga cga cac aag cat atg ttc aat ttc ctt gat
gtc aac cac aat gga aaa atc tct ctt gac gag atg gtc tac aag gca tct gat att
gtc atc aat aac ctt gga gca aca cct gag caa gcc aaa cga cac aaa gat gct gtG
gaa gcc ttc ttc gga gga gct gga atg aaa tat ggt gtg gaa act gat tgg cct gca
tat att gaa gga tgg aaa aaa ttg gct act gat gaa ttg gag aaa tac gcc aaa aac
gaa cca acC ctc atc cgC ata tgg ggt gat gct ttg ttt gat atc gtt gac aaa gat
caa aat gga gct att aca ctg gat gaa tgg aaa gca tac acc aaa gct gct ggt atc
atc caa tca tca gaa gat tgc gag gaa aca ttc aga gtg tgc gat att gat gaa agt
gga caa ctc gat gtt gat gag atg aca aga caG cat CtG gga ttt tgg tac acc atg
gat cct gct tgc gaa aag ctc tac ggt gga gct gtc ccc [SEQ ID NO: 9]

G4A

Atg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac
ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc
tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg
ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac
cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc
gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac
ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc
atg gcc gac aag cag aag aac ggc atc aag gCC aac ttc aag atc cgc cac aac atc
gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa
gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg
atc act cAc ggc atg gac gag ctg tac aag **tcc ggc ggg agc gga tcc ggc ggc cag**
tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc cag tcc
ggc ggg agc gga tcc ggc ggc cag tcc ggc ctc aGA TCT gtc aaa ctt aca tca gac
ttc gac aac cca aga tgg att gga cga cac aag cat atg ttc aat ttc ctt gat gtc
aac cac aat gga aaa atc tct ctt gac gag atg gtc tac aag gca tct gat att gtc
atc aat aac ctt gga gca aca cct gag caa gcc aaa cga cac aaa gat gct gtG gaa
gcc ttc ttc gga gga gct gga atg aaa tat ggt gtg gaa act gat tgg cct gca tat
att gaa gga tgg aaa aaa ttg gct act gat gaa ttg gag aaa tac gcc aaa aac gaa
cca acC ctc atc cgC ata tgg ggt gat gct ttg ttt gat atc gtt gac aaa gat caa
aat qga gct att aca ctg gat gaa tgg aaa gca tac acc aaa gct gct ggt atc atc
caa tca tca gaa gat tgc gag gaa aca ttc aga gtg tgc gat att gat gaa agt gga
caa ctc gat gtt gat gag atg aca aga caG cat CtG gga ttt tgg tac acc atg gat
cct gct tgc gaa aag ctc tac ggt gga gct gtc ccc [SEQ ID NO: 10]

G5A

Atg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac
ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc
tac ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg

ccc acc ctc gtg acc acc ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac
cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc
gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac
ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc
atg gcc gac aag cag aag aac ggc atc aag gCC aac ttc aag atc cgc cac aac atc
gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa
gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg
atc act cAc ggc atg gac gag ctg tac aag **tcc ggc ggg agc gga tcc ggc ggc cag**
tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc cag tcc
ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc
ctc aGA TCT gtc aaa ctt aca tca gac ttc gac aac cca aga tgg att gga cga cac
aag cat atg ttc aat ttc ctt gat gtc aac cac aat gga aaa atc tct ctt gac gag
atg gtc tac aag gca tct gat att gtc atc aat aac ctt gga gca aca cct gag caa
gcc aaa cga cac aaa gat gct gtG gaa gcc ttc ttc gga gga gct gga atg aaa tat
ggt gtg gaa act gat tgg cct gca tat att gaa gga tgg aaa aaa ttg gct act gat
gaa ttg gag aaa tac gcc aaa aac gaa cca acC ctc atc cgC ata tgg ggt gat gct
ttg ttt gat atc gtt gac aaa gat caa aat gga gct att aca ctg gat gaa tgg aaa
gca tac acc aaa gct gct ggt atc atc caa tca tca gaa gat tgc gag gaa aca ttc
aga gtg tgc gat att gat gaa aqt gga caa ctc gat gtt gat gag atg aca aga caG
cat CtG gga ttt tgg tac acc atg gat cct gct tgc gaa aag ctc tac ggt gga gct
gtc ccc [SEQ ID NO: 11]

SeG5A

Atg gtg agt gcc agt cgt cct gag gcc ctg gct gcc cct gtc acc act gtt gcg acc
ctt gtc cca cac aac gcc act gag cca gcc agt cct ggg gaa ggg aag gaa gat gcc
ttt tcc aag ctg aag cag aag ttt atg aat gaa ctg cat aaa atc cca ttg cca ccg
tgg gcc tta att gcc ata gcc ata gtt gcg gtc ctt cta gtc gtg acc tgc tgc ttc
tgt gtc tgt aag aaa tgt ttg ttc aaa aag aaa aac aag aag aag gga aag gaa aag
gga ggg aag aac gcc att aac atg aaa gac gtg aaa gac tta ggg aag acc atg aag
gat cag gcc ctt aag gat gac gat gct gaa act gga ctg act gat gga gaa gaa aag
gag gag ccc aag gaa gag gag aaa ctg gga aag ctt caa tat tca ctg gac tat gac
ttc cag aat aac cag ctg ctg gtg gga atc atc cag gct gct gaa ctg ccc gcc ctg
gac atg gga ggc aca tct gat cca tac gtc aaa gtc ttc ctg ctg ccc gac aaa aag
aag aag ttt gag aca aaa gtc cac cgg aaa acc ctc aat cca gtc ttc aat gaa cag
ttt act ttc aag gtg cca tac tcg gaa tta ggt ggc aag aca ctg gtg atg gct gtg
tat gat ttt gac cgc ttc tcc aag cac gac atc att gga gag ttc aaa gtt cct atg
aac acc gtg gat ttt ggc cac gtc acc gag gag tgg cgc gat ctc cag agt gct gag
aaa gaa gag caa gag aaa ctg ggt gac atc tgc ttc tcc ctc cgc tac gtc cct act
gcc ggc aag ctg act gtt gtc att ctg gaa gcc aag aac ctg aag aag atg gat gtg
qgt ggc tta tct gat ccc tat gta aag att cac ctg atg cag aac ggc aag aga ctg
aag aag aaa aag aca acg att aag aag aac aca ctt aac ccc tac tac aat gag tcc
ttc agc ttt gaa gtt ccg ttc gag caa atc cag aaa gtg caa gtg gtg gta act gtt
ttg gac tat gac aag att ggc aag aac gac gcc atc ggc aaa gtc ttt gtg ggc tac
aac agc acc ggc gca gag ctg cga cac tgg tca gac atg ctg gcc aac ccc cgg cgc
ccc atc gcc cag tgg cac act ctg cag gta gag gag gag gtt gat gcc atg ctg gct
gtc aag aGA tCC GGG AAT TCC GGG CGG gcc acc atg agc aag ggc gag gag ctg ttc
acc ggg gtg gtg ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc
agc gtg tcc ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc
atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg acc
tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag cag cac gac ttc ttc
aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac
ggc aac tac aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc
atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg
gag tac aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac ggc
atc aag gCC aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag ctc gcc
gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctq ctg ccc gac aac
cac tac ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac
atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act cAc ggc atg gac gag ctg
tac aag **tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc**

cag tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ggg agc gga tcc ggc ggc cag tcc ggc ctc aGA TCT gtc aaa ctt aca tca gac ttc gac aac cca aga tgg att gga cga cac aag cat atg ttc aat ttc ctt gat gtc aac cac aat gga aaa atc tct ctt gac gag atg gtc tac aag gca tct gat att gtc atc aat aac ctt gga gca aca cct gag caa gcc aaa cga cac aaa gat gct gtG gaa gcc ttc ttc gga gga gct gga atg aaa tat ggt gtg gaa act gat tgg cct gca tat att gaa gga tgg aaa aaa ttg gct act gat gaa ttg gag aaa tac gcc aaa aac gaa cca acC ctc atc cgC ata tgg ggt gat gct ttg ttt gat atc gtt gac aaa gat caa aat gga gct att aca ctg gat gaa tgg aaa gca tac acc aaa gct gct ggt atc atc caa tca tca gaa gat tgc gag gaa aca ttc aga gtq tgc gat att gat gaa agt gga caa ctc gat gtt gat gag atg aca aga caG cat CtG gga ttt tgg tac acc atg gat cct gct tgc gaa aag ctc tac ggt gga gct gtc ccc [SEQ ID NO: 12]

DNA sequence of GFP-aeguorin linkers

pGA (strain I2507) TCC GGC CTC AGA TCT [SEQ TD NO: 13]

pG1A (strain I2508) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 14]

pG2A (strain I2509) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 15]

pG4A (strain I2510) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 16]

pG5A (strain I2511) TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC GGG AGC GGA TCC GGC GGC CAG TCC GGC CTC AGA TCT [SEQ ID NO: 17]

pSeG5A (strain I2512) and pStG5A (strain I2513)same linker sequence than pG5A.

Peptide sequence of linkers

pGA Ser Gly Leu Arg Ser [SEQ ID NO: 18]

Pg1a Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 19]

pG2A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 20]

pG4A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 21]

pG5A Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Gly Ser Gly Ser Gly Gly Gln Ser Gly Leu Arg Ser [SEQ ID NO: 22]

pSeG5A and pStGSA idem than pG5A.

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Also incorporated by reference herein in its entirety is U.S. Patent 5,683,888.